

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
22 February 2001 (22.02.2001)

PCT

(10) International Publication Number
WO 01/12784 A1

- (51) International Patent Classification⁷: C12N 5/06, (74) Agent: BERNSTEIN, David, B.; Mintz, Levin, Cohn, Ferris, Glovsky and Popeo, P.C., One Financial Center, Boston, MA 02111 (US).
C07K 14/495, A61K 38/18, 38/19, 35/18
- (21) International Application Number: PCT/US00/22737
- (22) International Filing Date: 18 August 2000 (18.08.2000) (81) Designated States (*national*): AU, CA, JP.
- (25) Filing Language: English (84) Designated States (*regional*): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
- (26) Publication Language: English
- (30) Priority Data:
60/149,815 19 August 1999 (19.08.1999) US
09/637,539 11 August 2000 (11.08.2000) US
- (71) Applicant: NEW ENGLAND MEDICAL CENTER HOSPITAL [US/US]; Box 394, 750 Washington Street, Brookline, MA 02446 (US).
- (72) Inventor: BOHMER, Ralph, M.; 1038 Beacon Street #401, Boston, MA 02446 (US).
- Published:**
— With international search report.
— Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



WO 01/12784 A1

(54) Title: METHOD FOR TREATMENT OF β -HEMOGLOBINOPATHIES

(57) Abstract: The present invention provides a method for ameliorating β -globin disorders in a mammal. In one aspect of the invention, the treatment involves *ex vivo* treatment of early erythroid progenitor cells that leads to an increase in the relative amounts of cells subsequently expressing and accumulating HbF. The cell treatment is to be followed by transplantation of the modified cells. In another aspect of the invention, the same modification of progenitor cells occurs *in vivo*. Both treatments are based on the novel discovery that the modification can be performed very early in the erythroid maturation process, without disturbance of the subsequent proliferation and maturation of the erythroid precursor. The present invention also provides a procedure for the monitoring of β -globinopathies and the response of a patient to treatment. In this aspect of the invention, erythropoiesis of a patient is studied (in *vivo* or in *vitro*) by generating profiles of correlated contents of different types of hemoglobin present in nucleated red cells.

METHOD FOR TREATMENT OF β -HEMOGLOBINOPATHIES

FIELD OF THE INVENTION

The present invention relates generally to a method for treatment of β -hemoglobinopathies. More specifically, this invention relates to the treatment of β -hemoglobinopathies by administering a composition that promotes an increase in the relative amount of fetal erythropoiesis.

BACKGROUND OF THE INVENTION

Normal adult hemoglobin comprises four globin proteins, two of which are alpha (α) proteins and two of which are beta (β) proteins. During fetal development in mammals (particularly in humans) the fetus produces a fetal hemoglobin which comprises two gamma (γ)-globin proteins instead of the two β -globin proteins. At some point during fetal development or infancy, depending on the particular species and individual, there is a globin switch wherein the erythrocytes in the fetus switch from making predominantly γ -globin to making predominantly β -globin. The developmental switch from production of predominantly fetal hemoglobin (HbF, $\alpha_2\gamma_2$) to production of adult hemoglobin (HbA, $\alpha_2\beta_2$) occurs beginning at about 28 to 34 weeks of gestation and continues shortly after birth until HbA becomes predominant. This switch results primarily from decreased transcription of the gamma-globin genes and increased transcription of beta-globin genes. The blood of a normal adult contains only about 2% HbF.

Peripheral blood contains clonogenic cells that produce erythroid colonies and bursts in semisolid culture, given the appropriate combination of growth factors. Individual cells in such colonies can accumulate fetal hemoglobin (HbF), adult hemoglobin (HbA) or a combination of both. The pattern of hemoglobin expression and accumulation is different in cultures from fetal and adult blood. In cultures from adult blood, nucleated red cells accumulate either HbA (F-A+) only or a combination of HbF and HbA (F+A+).

Papayannopoulou, *et al.*, *Science* **199**: 1349-1350 (1978); Migliaccio, *et al.*, *Blood* **76**: 1150-1157 (1990). Individual colonies contain both F+ and F- cells, indicating that both

types are progeny from the same circulating stem cells. Thus, during the early stages of development in culture, cells execute an option whether or not to express HbF. The proportion of adult F+ cells developing in culture does not appear to be preprogrammed *in vivo*, but appears to depend on culture conditions: A shift into the combined HbF and HbA expression pathway can, for example, be achieved *in vitro* by high serum concentrations, due to the activity of an unidentified compound that can be absorbed on activated charcoal. Bohmer, *et al.*, *Prenatal Diagnosis* 19: 628-636 (1999); Migliaccio, *et al.*, *Blood* 76: 1150 (1990); Rosenblum, *et al.*, in: *Experimental Approaches for the Study of Hemoglobin* 397 (1985).

10 Hemoglobinopathies encompass a number of anemias of genetic origin in which there is a decreased production and/or increased destruction (hemolysis) of red blood cells (RBCs). There are genetic defects that result in the production by the body of abnormal hemoglobins with a concomitant impaired ability to maintain oxygen concentration. Some such disorders involve the failure to produce normal β -globin in sufficient amounts, some
15 involve the failure to produce normal β -globin entirely. These disorders associated with the β -globin protein are referred to generally as β -hemoglobinopathies. For example, β -thalassemias result from a partial or complete defect in the expression of the β -globin gene, leading to deficient or absent HbA; sickle cell anemia results from a point mutation in the β -globin structural gene, leading to the production of an abnormal (sickled) hemoglobin
20 (HbS).

Sickle cell anemia (sickle cell disease, SCD) is an inherited, chronic, hemolytic anemia characterized by sickle-shaped RBCs. Because deoxygenated HbS is much less soluble than deoxy HbA, it forms a semisolid gel of rod-like tactoids, causing the RBCs to assume a sickle shape. HbS RBCs are more fragile than normal RBCs and hemolyze more
25 readily, leading eventually to anemia.

It has been observed that certain populations of adult patients with beta chain abnormalities have higher than normal levels of fetal hemoglobin (HbF) and have been observed to have a milder clinical course of disease than patients with normal adult levels of HbF. For example, a group of Saudi Arabian sickle-cell anemia patients who express 20-
30 30% HbF have only mild clinical manifestations of the disease. Pembrey, *et al.*, *Br. J. Haematol.* 40: 415-429 (1978). There are also a variety of distinct genetic mutations that

cause hereditary persistence of HbF, in which gamma-globin gene expression is not downregulated during development. This condition has been shown to significantly decrease the severity of sickle cell anemia or β -thalassemia in individuals simultaneously affected with both traits. Wood and Weatherall, *Biochem J.* **215**: 1-10 (1983). It is now
5 accepted that hemoglobin disorders, such as sickle cell anemia and the β -thalassemias, are ameliorated by increased HbF production. Reviewed in Jane and Cunningham *Br. J. Haematol.* **102**: 415-422 (1998). See, also, Bunn, *N. Engl. J. Med.* **328**: 129-131 (1993).

While the developmental switch from gamma— to beta—globin gene expression is strictly controlled, there is evidence that external factors can influence gamma-globin gene
10 expression. For example, a delay in the fetal to adult hemoglobin switch has been observed in infants of diabetic mothers, suggesting an affect by circulating physiological factors. Perrine, *et al.*, *N. Engl. J. Med.* **312**: 334-338 (1985). Additionally, the ability to enhance HbF synthesis *in vivo* by pharmacological manipulation was demonstrated in baboons treated with 5-azacytidine (5-AzaC). DeSimone, *et al.*, *Proc. Natl. Acad. Sci. USA* **79**:
15 4428-4431 (1982). Subsequent studies confirmed the ability of 5-AzaC to increase HbF in patients with β -thalassemia and sickle cell disease. Ley, *et al.*, *N. Engl. J. Medicine*, **307**: 1469-1475 (1982), and Ley, *et al.*, *Blood* **62**: 370-380 (1983).

Other agents that stimulate HbF *in vivo* include hydroxyurea [Carache, *et al.*, *N. Engl. J. Med.* **332**: 1317-1322 (1995)], butyrates [Perrine, *et al.*, *N. Engl. J. Med.* **328**: 81-86
20 (1993); Perrine, *et al.*, *Am. J. Pediatr. Hematol. Oncol.* **16**: 67-71 (1994)], activin and inhibin (US Patent 4,997,815), and various organic acids (*e.g.* valeric, polyhydroxy-benzoic, phenylacetic, mandelic) See, *e.g.*, U.S. Patent Nos. 5,366,996 and 5,700,640. Although these agents act *via* mechanisms that are not yet completely understood, it is thought that they partially derepress gamma-globin gene expression, leading to increased levels of HbF.

25 The effectiveness of many of these therapeutic agents has been demonstrated in several clinical trials, but is limited by unwanted side effects and variability in patient responses. Jane and Cunningham, *Br. J. Haematol.* **102**: 415-422 (1998); Olivieri, *Seminars in Hematology* **33**: 24-42 (1996). For example, very high dosages of butyric acid are necessary for inducing gamma-globin gene expression, requiring catheterization for
30 continuous infusion of the compound. Moreover, these high dosages of butyric acid can be associated with neurotoxicity and multiorgan damage. Blau, *et al.*, *Blood* **81**: 529-537

(1993). There are also limitations to the therapeutic use of hydroxyurea; potential long-term consequences of treatment with this compound include teratogenic and oncogenic effects. While even minimal increases in HbF levels are helpful in sickle cell disease, β -thalassemias require a much higher increase that is not reliably, or safely, achieved by any
5 of the currently used agents. Olivieri, *Seminars in Hematology* 33: 24-42 (1996).

Thus, a need remains in the art for additional, novel, therapeutic methods for treatment of β -hemoglobinopathies — with reduced toxicity— capable of sustained induction of HbF .

SUMMARY OF THE INVENTION

10 The present invention provides a method for ameliorating β -globin disorders in a mammal. In one aspect of the invention, the treatment involves *ex vivo* treatment of early erythroid progenitor cells that leads to an increase in the relative amounts of cells subsequently expressing and accumulating HbF. The cell treatment is to be followed by transplantation of the modified cells. In another aspect of the invention, the same
15 modification of progenitor cells occurs *in vivo*. Both treatments are based on the novel discovery that the modification can be performed very early in the erythroid maturation process, without disturbance of the subsequent proliferation and maturation of the erythrocyte. The present invention also provides a procedure for the monitoring of β -globinopathies and the response of a patient to treatment. In this aspect of the invention,
20 erythropoiesis of a patient is studied (in *vivo* or in *vitro*) by generating profiles of correlated contents of different types of hemoglobin present in nucleated red cells (*e.g.* HbA vs. HbF, HbF vs. HbS, or HbS vs. HbA profiles).

BRIEF DESCRIPTION OF THE DRAWINGS

25 FIG. 1 depicts two-color flow cytometry profiles of correlated cellular hemoglobin contents after treatment with 30% FCS or 10 ng/ ml rhTGF-beta 1. Comparison between continuous exposure (day 0-7, left column, graphs A, B, C) and exposure for the first 4 days of culture (day 0-4, right column, graphs D, E, F). Analysis was on day 7 of culture. Cultures were mixed into single-cell suspensions and the correlated contents of HbF and HbA for each

individual cell measured by . Each analyzed cell sample results from a mixture of about 100 colonies. Each profile results from 10,000 intact nucleated cells with normal DNA content, gated by Hoechst fluorescence. The proportions of F+ cells (percent of all Hb+ cells) are indicated in the upper right corner of each profile.

FIG. 2 is a graph showing stimulation of HbF by short-term incubation with FCS or TGF-beta. Cultures initiated in the presence of TGF-beta or FCS were washed and re-seeded in control medium after 0, 1, 3 and 4 days. On day 7 of culture, the proportions of F+ cells were determined as a function of exposure time. Circles: control; squares: FCS; triangles: TGF-beta.

FIG. 3 is a graph depicting the statistics of the effect of TGF-beta 1 on cultures from different donors, comparing various treatment timings between days 0 and 5. The median values are indicated as horizontal bars. Each individual case is displayed by an open circle. In the case of 4-day treatment, data pairs are connected to demonstrate the correlation of variations ($r = +0.84$). The difference between controls and 4-day TGF-beta treatment is statistically highly significant ($p < 0.0001$).

FIG. 4 depicts a graph of the proportions of F+ cells at a later culture phase. Cultures were incubated with TGF-beta or FCS for 4 days, then washed and re-seeded in fresh control medium. On days 7 and 10, the cultures were further diluted in fresh control medium (closed symbols) or medium supplemented with TGF-beta 1 (open symbols). The percent F+ cells were determined at selected times between day 7 and day 13. The time course is shown from one experiment where all conditions were investigated together on the cells from the same donor. Part of this experiment was repeated 3 more times, comparing day 0-4 TGF-beta treatment and control. The ranges of resulting F+ proportions, measured on days 7, 10 and 13, are indicated in the figure as vertical bars.

FIG. 5 is a graph depicting the selective deletion of F-A+ cells. Cultures were treated with TGF-beta for 4 days, then propagated without TGF-beta, with further subcultivation and dilution on days 7 (1:10), 10 (1:5) and 13 (1:5).

Absolute numbers of F+ and F-A+ cells per culture were determined on days 7, 10, 13 and 16.

FIG. 5A is an example of a time course from one experiment. Total cell numbers were calculated using preceding culture dilution factors in the following manner: at each time of analysis, some of the cells were removed for counting, those that go back into the culture after counting are seeded with a dilution of fresh medium, to avoid medium exhaustion. Therefore, the actual numbers in the plates increase only between seeding and harvest. To calculate cell production over the whole culture time, it is necessary to multiply the counted cell numbers in the plates with all previous culture dilution factors. Closed symbols depict data from F-A+ cells, open symbols show F+ cells, and triangles show TGF-beta-treated cultures, while circles show the controls.

FIG. 5B is a graph indicating the TGF-beta -induced change of total F+ and F- cell numbers, shown as ratio of $N(\text{TGF}) / N(\text{CON})$. Individual experiments are distinguished by different symbols. Pairs of F+ and F-A+ values are connected to demonstrate positive correlation. Median values are indicated by horizontal bar.

FIG. 6 shows two-color flow cytometry profiles demonstrating the effect of different cytokine combinations on the proportions of F+ cells and the effect of TGF-beta. Cultures were grown in media with combinations of cytokines as indicated on the right side of the profiles, in the presence (TGF) or absence (CON) of TGF-beta. After 4 days, cultures were re-seeded without TGF-beta in fresh media with all three cytokines. Profiles were recorded after 8 days of culture (4 days after re-seeding).

FIG. 7 is a graph demonstrating the effect of TGF-beta on the cell cycle during short-term treatment. Cell cultures were seeded in the presence (TGF) and absence (CON) of TGF-beta and on days 1, 2, 3 and 4 washed and re-seeded in the absence of TGF-beta. On day 9, the secondary colonies/clusters were counted and plotted as a function of the number of the day of treatment

before reseeding. The insert shows the degree of colony reduction $[n(\text{TGF}) / n(\text{CON})]$ by 4-day TGF treatment. Statistics from 5 experiments are shown.

FIG. 8 depicts a graph of titrations of different forms of TGF-beta (beta 1, 2 and 3). Cultures were exposed to TGF-beta 1, 2 and 3 at a wide range of concentrations. On day 4, cultures were re-seeded in control medium, and the proportions of F+ cells were determined on day 7.

FIG. 9 shows two-color flow cytometry hemoglobin profiles from a sickle cell patient before culture (FIG. 9A) and after 7 days of culture in control medium (FIG. 9B), FCS (FIG. 9C) and TGF-beta (FIG. 9D).

FIG. 10. is a graph demonstrating that cells programmed to turn into F-A+ cells are being suppressed earlier in the culture phase compared to cells programmed to become F+ cells. Cultures of adult mononuclear blood cells were seeded with TGF-beta1 (10ng/ml) and re-seeded in the absence of TGF-beta on the day indicated. On day 8, the absolute numbers of F+ and F-A+ cells were determined and normalized to the values without TGF-beta treatment.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for ameliorating β -globin disorders in a mammal by treatment of patients with their own stem cells, or other, immunologically compatible stem cells, modified to generate increased proportions of HbF cells. The HbF initiation methods described herein are based on the discovery that there is a narrow time window early after mitogenic stimulation of quiescent cells (between day 2 and day 4 in culture) where said modification takes effect. Due to the HbF initiation early in the differentiation cascade, initiated stem cells retain the potential to produce large numbers of mature red blood cells.

In one aspect of the invention, the treatment involves *ex vivo* stimulation of HbF production, followed by transplantation of stimulated cells. In this procedure, a patient's hemopoietic stem cells are cultured with the appropriate cytokines, according to procedures well-known in the art (e.g., density gradient centrifugation followed by optional CD34+ enrichment and placement of the stem cell preparations into a standard hemopoietic cell

culture medium). In one embodiment of the invention, the stem cells are collected from the umbilical cord blood of a β -globinopathy patient at birth. In another embodiment, the source of stem cells is peripheral blood collected at later stages of a patient's life. In yet another embodiment of the present invention, the stem cells are collected from the bone marrow of a patient. While the use of autologous stem cells is preferable, the methods of this invention include the use of stem cells from other sources that are immunologically compatible.

The cultured stem cells are exposed to an agent that causes an increase in the proportions of cells expressing and accumulating HbF, by mechanisms not yet fully understood. They may include a reversal of the hemoglobin switch or a selective elimination of cells programmed to express only HbA. In one embodiment, the agent is a preparation of human cord serum, either preferentially derived from the patient at birth, or from other sources. This serum is specifically treated as described. In another embodiment, the agent is recombinant TGF-beta1, 2 or 3. The *in vitro*-treated cells are immature enough to re-populate bone marrow and contribute to the patient's erythropoiesis upon transplantation. In one aspect of the invention, the cells are transplanted directly upon removal from culture. In another aspect of the invention, prior to hematopoietic reconstitution, the cells are cryopreserved according to techniques common in the art, such as describe in U.S. Patent No. 5,192,553.

In another aspect of the invention, the stimulation of HbF production occurs *in vivo*. An agent capable of modifying F+ cell proportions is injected into a β -hemoglobinopathy patient. In one embodiment of this invention, TGF-beta is injected into the patient. This treatment is performed at suitable intervals that do not suppress the patient's overall hemopoiesis. In another embodiment *in vivo* small molecule derivatives of TGF-beta, or an agent having properties similar to TGF-beta, or parts of such derivatives, trigger the receptors and subsequent responses that are responsible for the mechanism by which TGF-beta causes the HbF increase. Such derivatives of TGF-beta may have decreased side effects, i.e., specific beneficial and non-beneficial effects of TGF-beta may be dissociated since TGF-beta appears to have many receptors with different activities. TGF-beta is generally present in body tissues in abundance. It is, however, normally present in a latent, deactivated form through its association with other molecules, such as latency-associated

peptide (LAP). TGF-beta may become activated in tissues on demand. Thus, in another aspect of the invention, rather than injecting active TGF-beta, latent TGF-beta in body tissues may be activated by the injection of a specific agent into a patient. Such agent may, for example, be one that dissociates a latency-associated peptide (LAP) from TGF.

5 The present invention also provides a procedure for monitoring β -globinopathies and the response of a patient to treatment. In this aspect of the invention, erythropoiesis of a patient is studied *in vitro* or *in vivo* by generating flow cytometric profiles of correlated contents of different types of hemoglobin present in the nucleated or non-nucleated red blood cells (*e.g.* HbA vs. HbF; HbF vs. HbS; or HbS vs. HbA profiles). Bohmer, *et al.*, *Br. J. Haematol.* 103: 351-360 (1998).

Example 1 Two-Parameter Flow Cytometry

 The present invention is directed to a novel method of quantitative two-color flow cytometry of fluorescently labeled cells. Cells are labeled simultaneously with different fluorescence-conjugated antibodies specific to the type(s) of hemoglobin present in the cell.

15 Antibodies to HbF (gamma-globin chain), HbA (beta-globin chain), and to HbS (-beta-globin chain with the sickle mutation) are used. The profiles of correlated hemoglobin contents are analyzed. This method provides information not available with single-parameter hemoglobin measurements. Bohmer, *et al.*, *Prenatal Diagnosis*, and *Br.J.Haematol.*

20 1.1 Cell Labeling

 Cells are fixed with 5% formaldehyde in PBS at 37 °C for 1 h, exposed to 100% methanol for 5 min at room temperature, then permeabilized in Solution B of the Caltag Fix & Perm kit during incubation with phycoerythrin-conjugated antibodies to the gamma chain of hemoglobin (HbF) (Cortex) and fluorescein isothiocyanate-conjugated antibodies to the

25 beta chain of hemoglobin (HbA) or to fluorescein isothiocyanate -conjugated antibodies specific for sickle cell hemoglobin (HbS) (Isolabs). After incubation, cells are washed and suspended in PBS with 1% formaldehyde and 0.1 μ g/ml Hoechst 33342.

1.2 Flow Cytometry

Cells are processed in a Becton-Dickinson Vantage flow cytometer/cell sorter with dual laser excitation (UV and 488 nm). Intact nucleated cells are selected by gating on UV-excited Hoechst 33324 fluorescence (430 nm) which yielded DNA histograms as well as cell cycle information by BudR-induced fluorescence quenching. Bohmer, *Cell Tissue Kinet.* 12: 101-110 (1979). Phycoerythrin (PE) and Fluorescein isothiocyanate (FITC) are excited at 488 nm and measured at 530nm and 575nm, respectively. Correlated fluorescence values are recorded with appropriate color compensation. The accuracy of color compensation over 4 logs of fluorescence values is limited. To obtain absolute cell counts from the relative particle counts that a flow cytometer provides, known amounts of fluorescent plastic beads (Coulter Immunobrite Level IV) are added to the cell suspensions. Bohmer, *Cell Tissue Kinet.* 17: 593-600 (1984).

Example 2 *Ex vivo* modification of early erythroid stem cells to increase the proportions of developing F+ cells

2.1 Cell Culture

Blood samples were kept at room temperature and processed as soon as possible (between 2h and 24h) after collection. The blood was diluted 1:4 with phosphate buffered saline (PBS), the mononuclear cells isolated by density gradient (density 1.077), washed in PBS with 1% BSA (bovine serum albumin) and cultured without further processing in standard 6-well plates, 3-5 ml per well, at a maximum density of 0.3 million/ml. The standard medium (referred to as "control" medium) comprised a mixture of 2/3 Iscoves MDM and 1/3 RPMI1640, containing methylcellulose (0.9%), C-CHS (1%), EPO (1 U/ml), SCF (20 ng/ml), IL3 (10 ng/ml), insulin (3 µg/ml), iron-saturated transferrin (70 µg/ml), mercaptoethanol (0.7 nM). Cytokines were from R&D Systems, Minneapolis, MN or Genzyme, Cambridge, MA.

2.2 Autologous serum preparation

Human cord blood was collected without anticoagulant, the clots removed, the remaining blood centrifuged at 3000 rpm for 20 min., and the supernatant serum was collected. Approximately 10 ml is a suitable quantity of serum for one cell culture

treatment. The serum was extracted twice with 3 volumes (*ca.* 30 ml) of chloroform. This removes toxic substances and allows the serum to be used at high concentrations.

5 **2.3. Cell culture treatment with serum**

On day 1 or day 2 of culture, the chloroform-extracted serum was added to the stem cell cultures at 30% of the total culture medium volume. After 2 or 3 days of culture (day 4 overall), the cells were removed from the culture and washed. The cells were then ready for transplantation or cryopreservation.

10 **2.4 TGF-beta treatment of cultures**

Transforming growth factor beta (TGF-beta) was found to be more powerful than serum, when used with the same timing. A relatively brief exposure to TGF-beta during the first few days of cell culture caused a lasting shift towards HbF expression with no reduction in subsequent clonal expansion (see data below). This effect did not depend on
15 the combination of cytokines that were used to stimulate and support erythropoiesis. Mononuclear cells from adult blood were cultured from day 0 to day 4 with 10 ng/ml recombinant human transforming growth factor beta-1, hereinafter referred to as rhTGF-beta-1 or TGF-beta. The most useful concentration of TGF-beta depended on cell density and timing of exposure.

20 **(a) Increase in F+ cell proportions by short-term TGF-beta treatment**

The effect of TGF-beta treatment on cell cultures was examined and compared to the effect of serum. Cultures were incubated with TGF-beta or serum from day 0 to day 4 of culture. On day 4, part of the cultures were washed and re-seeded in control medium without TGF-beta or serum, the other part remained unmodified until the time of harvest.
25 TGF-beta was removed from cultures by washing the cultures twice in PBS/BSA, then re-seeding them in fresh medium. On day 7, cultures containing hundreds of colonies were turned into single-cell suspensions and the correlated contents of HbA and HbF measured by flow cytometry (FIG. 1). In the control cultures, the large majority of nucleated red cells were clustered at high levels of HbA but little or no HbF (F-A+). A small proportion (*ca.*

20% in this case) contained HbF together with HbA (F+A+). Some cells were F+A- or spread over all areas of the profile. The subdivision of profiles chosen for numerical evaluations is indicated in the first profile. The time course of hemoglobin synthesis in erythroid cultures is discussed in Bohmer, *et al.*, *Br. J. Haematol.* **103**: 351-360 (1998),

5 herein incorporated by reference. TGF-beta increased the proportions of F+ cells dramatically, and more so than serum. Four days of exposure to the HbF-inducing agents appeared nearly as effective as continuous exposure up to the day of analysis (day 7). Continuous TGF-beta treatment, but not the 4-day treatment, resulted in strongly reduced total cell counts, and colonies were much smaller and appeared to contain apoptotic/
10 necrotic cells. Various additional tests showed that the effect of serum is not based on active TGF-beta in serum. The combination of TGF-beta with 30% treated serum gives an even better result than either of the two agents alone, with reduced toxicity and a near 100% F+ population.

(b) Induction of HbF expression within the first four days of culture

15 The proportions of F+ cells as a function of time of exposure to TGF-beta are shown in FIG. 2. Also included on this graph are data from cells cultured in 30% fetal calf serum (FCS). The proportions of F+ cells began to increase between days 1 and 2 and nearly leveled out by day 4. The proportions of F+ cells reached a plateau at *ca.* 50% with FCS and 80% with TGF-beta. The time course of TGF and serum effects were similar.

20 To test if the required exposure time could be further narrowed, cultures were exposed to TGF-beta for only 1 day during the first 5 days of culture (FIG. 3). Following exposure for only one day between days 2 and 4, the proportions of F+ cells were substantially increased and only slightly less than after exposure from day 0 to day 4.

(c) F+ cell proportions at the later culture phase

25 The proportions of F+ cells during further culture development were monitored in a separate experiment (FIG. 4). After a 4-day treatment with TGF-beta or serum, cultures were grown in control medium and again re-seeded on days 7 and 10, each time with a 10-fold dilution, to minimize the exhaustion of medium components by the rapidly expanding cell mass. The increased proportions of F+ cells, as introduced by brief TGF-beta or serum

treatment, were maintained during further culture growth without TGF-beta or serum, suggesting that F+ and F-A+ cells expanded at the same rate.

To assess the effect of TGF-beta at this later culture phase, parts of the cultures in the same experiment were newly supplied with TGF-beta beginning on day 7 (data included with open symbols in FIG. 4). When added at this time, TGF-beta had no effect on F+ proportions. However, a complete and lasting growth arrest in the newly developing secondary colonies occurred after approximately 2 days of TGF-beta treatment, mostly at the 8-16 -cell stage (data not shown).

(d) Effect of TGF-beta on absolute cell numbers and long-term proliferation

The increase in the proportions of F+ cells measured between week 1 and 2 of culture could be due to several different mechanisms, such as a reversal of the hemoglobin switch, a selective proliferative boost to F+ cells, or a selective inhibition of F-A+ cells. If the TGF-beta effect were to be based on purely one of these three mechanisms, it could be distinguished by quantitating absolute, instead of relative, cell numbers. A reversal of the hemoglobin switch would lead to increased numbers of F+ cells at the numerically equal expense of F-A+ cells. A selective increase in the rate of F+ cell proliferation would leave F-A+ cell numbers unchanged. Similarly, a selective suppression of F-A+ cells would leave F+ cell numbers unchanged.

Cultures that were treated with TGF-beta from day 0-4 were sub-cultivated again on days 7, 10 and 13, and the total numbers of F+ and F-A+ cells per culture were determined on days 7, 10, 13 and 16. FIG. 5A shows the time course from a representative experiment. Between days 7 and 10, the total numbers of F+ cells (open symbols) in TGF-beta -treated and control cultures were approximately equal, whereas F-A+ cells (full symbols) were dramatically reduced in the TGF-beta -treated cultures. Between day 7 and 10, both cell types in both cultures proliferated at approximately the same rate. After day 10, in control cultures, the proliferation decreased strongly and equally for both F+ and F-A+ cells, maintaining the ratio. No secondary colonies were seen following the subcultivation on day 13. In contrast, the proliferation in TGF-beta -treated cultures continued for much longer for both F+ and F-A+ cells. The F-A+ cell numbers were gradually reducing the gap with F+ cells, in agreement with the gradually decreasing proportions of F+ cells shown in FIG.

4. The number of days for which the proliferation of TGF-beta -treated cultures could be maintained was highly variable between donors and appeared to correlate with the initial numbers of clonogenic cells per ml of blood. The complete cessation of proliferation around day 13 in untreated control cultures occurred with little variation between donors.

5 In spite of variations in the exact time course and culture lifespan, both the initial reduction of F-A+ cells and the increase in F+ division potential was strictly reproduced in 4 experiments from different donors, and the data are summarized in FIG. 5B. The ratios of absolute cell numbers in TGF-beta -treated and control cultures ($N(\text{TGF})/N(\text{CON})$) are shown on different days, with medians indicated as horizontal bars, the pairs of F+ and F-A+ values connected and the 4 individual cases distinguished by symbol. On day 7, the F+ cell numbers were little affected by TGF-beta (ratio = 1), whereas the F-A+ cell numbers were reduced *ca.* 10-fold. The day-7 values are available from 12 additional experiments (not shown), with medians exactly the same as shown here for the 4 experiments where the entire culture lifespan was studied. With increasing culture time, the ratios increased for 15 both F+ and for F-A+ cells, indicating that both types of cells in TGF-beta -treated cultures were able to out-proliferate the controls, as exemplified in FIG.5A. On average, the F+ population of TGF-beta -treated cultures grew to nearly 10-fold higher levels than the controls. However in 2 (out of 5) experiments, the TGF-beta -treated cultures kept proliferating and producing secondary colonies beyond 3 weeks, with $N(\text{TGF})/N(\text{CON})$ of 20 F+ cells exceeding 100. Under standard culture conditions, this lifespan is usually observed only in cultures from fetal blood at early gestational age.

(e) TGF-beta induction does not depend on the combination of cytokines

TGF-beta may interact with the signaling mechanisms of cytokines required to support erythropoiesis. The manner in which HbF stimulation by TGF-beta would be 25 affected by different cytokine cocktails that are known to support erythropoiesis less than optimally was examined. Cultures were initiated in EPO+SCF+IL3, EPO+SCF, EPO+IL3 and SCF+IL3, and treated with TGF-beta 1 for the first 4 days. The cultures were then re-seeded, without TGF-beta, in the full cytokine cocktail (EPO+SCF+IL3) and analyzed between days 7 and 9 of culture. FIG. 6 shows examples of the resulting profiles. While the 30 shape of hemoglobin profiles as well as the total numbers of hemoglobinized cells were

affected by sub—optimal cytokine combinations, the TGF-beta-induced increase of F+ cells was similar for all conditions.

(f) Minimal cell cycle perturbation during short-term TGF-beta treatment

To test the cell cycle effect of TGF-beta during the first 4 days of treatment, the numbers of secondary erythroid colonies that developed upon re-seeding the cultures after different duration of TGF-beta exposure were counted (FIG. 7). A decrease in the number of secondary colonies upon re-seeding would reflect a decrease in the number of divisions undergone by colony forming cells before the cultures were re-seeded as a single-cell suspension. However, the secondary colonies became smaller and more numerous, without much effect of TGF-beta. The numbers of secondary colonies began to increase between day 1 and 2, equally in TGF-beta and controls. Between days 3 and 4 the beginnings of inhibition by TGF-beta could be seen. The degree of inhibition (decrease in secondary colony numbers) beginning between day 3 and 4 of TGF-beta exposure was variable between experiments and did not strictly correlate with the resulting proportions of F+ cells. Thus, we do not yet fully control this mechanism. We conclude that TGF-beta did not interfere with cytokine-dependent proliferation during the first few days of treatment.

(g) Relative effectiveness of different forms of TGF-beta

To investigate the relative potencies of different forms of TGF-beta to increase the proportions of F+ cells, TGF-beta 1, 2 and 3 were titrated over a wide range of concentrations. The averaged data of two experiments with blood cells from different donors are shown in FIG. 8. For these titrations, treatment began on day 2, the onset of the TGF-sensitive culture phase. At this time (day 2), the titration curves shifted to much lower concentrations compared to treatments from day 0-4. This shift may be due to TGF degradation starting at the time of addition to the culture. TGF-beta 2 was nearly 100 times less effective than TGF-beta 1. The relative potency of TGF-beta 3 was in between TGF-beta 1 and TGF-beta 2.

(h) Effect of TGF-beta and FCS on sickle cell erythropoiesis

Mononuclear cells from one sickle cell patient were continuously cultured in 30% FCS or 10 ng/ml TGF-beta. The cells were labeled with the same antibodies to HbF but

with antibodies specific for HbS instead of HbA. FIG. 9 shows the hemoglobin profiles on day 7 of culture, the profile before culturing is also shown in FIG. 9A, representing nucleated red cells circulating in the peripheral blood of the patient. Both serum and TGF-beta caused an increase in relative F+ cell numbers, similar to the trend seen in normal blood cultures.

EQUIVALENTS

From the foregoing detailed description of the specific embodiments of the invention, it should be apparent that a unique method of treating β -hemoglobinopathies has been described. Although particular embodiments have been disclosed herein in detail, this has been done by way of example for purposes of illustration only, and is not intended to be limiting with respect to the scope of the appended claims that follow. In particular, it is contemplated by the inventor that various substitutions, alterations, and modifications may be made to the invention without departing from the spirit and scope of the invention as defined by the claims

CLAIMS

What is claimed is:

1. A method for the treatment of a β -hemoglobinopathy in a subject, the method comprising the steps of:

5 (a) providing a culture comprising erythroid progenitor cells and cytokines, wherein said cytokines are present in an amount sufficient to increase the number of fetal hemoglobin producing erythroid cells in said culture; and

(b) introducing treated cells from said culture into said subject,
thereby treating said β -hemoglobinopathy in said subject.

10

2. The method as recited in claim 1, wherein said step of providing said culture of erythroid progenitor cells further comprises the step of contacting said culture with one or more agents in an amount sufficient to increase production of HbF in said erythroid progenitor cells.

15

3. The method as recited in claim 1, wherein said erythroid progenitor cells are derived from said subject.

4. The method as recited in claim 3, wherein said subject is neonatal.

20

5. The method as recited in claim 3, wherein said erythroid progenitor cells are autologous hematopoietic stem cells.

6. The method as recited in claim 5, wherein said autologous hematopoietic stem
25 cells are derived from umbilical cord blood.

7. The method as recited in claim 5, wherein said autologous hematopoietic stem cells are obtained from peripheral blood.

8. The method as recited in claim 5, wherein said autologous hematopoietic stem
5 cells are obtained from bone marrow.

9. The method as recited in claim 1, wherein said erythroid progenitor cells are non-autologous stem cells.

10. The method as recited in claim 2, wherein at least one of said agents is present
10 in human cord serum.

11. The method as recited in claim 2, wherein at least one of said agents is transforming growth factor beta (TGF-beta).

15

12. The method as recited in claim 1, wherein said step of introducing said treated cells into said subject occurs immediately upon the removal of said treated cells from said culture.

13. The method as recited in claim 1, wherein said culture is provided as a preserved
20 culture.

14. The method as recited in claim 13, wherein said culture is provided as a cryopreserved culture.

15. The method as recited in claim 1, wherein said culture is provided *ex vivo*.
25

16. The method as recited in claim 2, wherein said agent is a cytokine.

17. The method as recited in claim 1, wherein said subject is a mammal.

18. The method as recited in claim 1, wherein said subject is a human.

5

19. A method for stimulating the production of HbF cells, the method which comprises providing a culture comprising erythroid progenitor cells and cytokines, wherein said cytokines are present in an amount sufficient to increase the number of fetal hemoglobin producing erythroid cells in said culture

10

20. The method as recited in claim 19, wherein said step of providing said culture of erythroid progenitor cells further comprises the step of contacting said culture with one or more agents in an amount sufficient to increase production of HbF in said erythroid progenitor cells.

15

21. The method as recited in claim 19, wherein said erythroid progenitor cells are derived from said subject.

22. The method as recited in claim 21, wherein said subject is neonatal.

20

23. The method as recited in claim 21, wherein said erythroid progenitor cells are autologous hematopoietic stem cells.

24. The method as recited in claim 23, wherein said autologous hematopoietic stem
25 cells are derived from umbilical cord blood.

25. The method as recited in claim 23, wherein said autologous hematopoietic stem cells are obtained from peripheral blood.

26. The method as recited in claim 23, wherein said autologous hematopoietic stem
5 cells are obtained from bone marrow.

27. The method as recited in claim 19, wherein said erythroid progenitor cells are non-autologous stem cells.

10 28. The method as recited in claim 20, wherein at least one of said agents is present in human cord serum.

29. The method as recited in claim 20, wherein at least one of said agents is transforming growth factor beta (TGF-beta).

15

30. The method as recited in claim 19, wherein said culture is provided as a preserved culture.

31. The method as recited in claim 30, wherein said culture is provided as a
20 cryopreserved culture.

32. The method as recited in claim 19, wherein said culture is provided *ex vivo*.

33. The method as recited in claim 20, wherein said agent is a cytokine.

25

34. The method as recited in claim 19, further comprising the step of introducing treated cells from said culture into a subject.

35. The method as recited in claim 19, wherein said subject is a mammal.

36. The method as recited in claim 19, wherein said subject is a human.

5

37. A method for increasing the proportion of cells expressing and accumulating HbF, the method comprising the steps of:

(a) preparing a culture comprised of erythroid progenitor cells, cytokines, and one or more agents in an amount sufficient to increase the proportions of cells which express and
10 accumulate HbF; and

(b) introducing treated cells from said culture into a subject.

38. The method as recited in claim 37, wherein said erythroid progenitor cells are derived from said subject.

15

39. The method as recited in claim 38, wherein said subject is neonatal.

40. The method as recited in claim 37, wherein said erythroid progenitor cells are autologous hematopoietic stem cells.

20

41. The method as recited in claim 40, wherein said autologous hematopoietic stem cells are derived from umbilical cord blood.

42. The method as recited in claim 40, wherein said autologous hematopoietic stem
25 cells are obtained from peripheral blood.

43. The method as recited in claim 40, wherein said autologous hematopoietic stem cells are obtained from bone marrow.

5 44. The method as recited in claim 37, wherein said erythroid progenitor cells are non-autologous stem cells.

45. The method as recited in claim 37, wherein at least one of said agents is present in human cord serum.

10 46. The method as recited in claim 37, wherein at least one of said agents is transforming growth factor beta (TGF-beta).

15 47. The method as recited in claim 37, wherein said step of introducing said treated cells into said subject occurs immediately upon the removal of said treated cells from said from said culture.

48. The method as recited in claim 37, wherein said culture is provided as a preserved culture.

20 49. The method as recited in claim 37, wherein said culture is provided as a cryopreserved culture.

50. The method as recited in claim 37, wherein said culture is provided *ex vivo*.

25 51. The method as recited in claim 37, wherein said agent is a cytokine.

52. The method as recited in claim 37, wherein said subject is a mammal.

53. The method as recited in claim 37, wherein said subject is a human.

54. A method for stimulating production of HbF cells in a subject, the method
5 which comprises administering to said subject one or more agents capable of modifying fetal Hb containing cells in said subject.

55. The method as recited in claim 54, wherein at least one of said agents comprises transforming growth factor beta (TGF-beta).

10

56. A method for the treatment of a β -globin disorder in a subject, the method comprising the steps of:

(a) providing a culture comprising erythroid progenitor cells and cytokines, wherein said cytokines are present in an amount sufficient to increase the number of fetal
15 hemoglobin producing erythroid cells in said culture;

(b) exposing said culture to one or more agents in an amount that increases the proportions of cells expressing and accumulating HbF; and

(c) introducing treated cells from said culture into said subject,
thereby treating said β -globin disorder in said subject.

20

57. The method as recited in claim 56, wherein said erythroid progenitor cells are derived from said subject.

58. The method as recited in claim 57, wherein said subject is neonatal.

25

59. The method as recited in claim 57, wherein said erythroid progenitor cells are autologous hematopoietic stem cells.

60. The method as recited in claim 59, wherein said autologous hematopoietic stem cells are derived from umbilical cord blood.

5 61. The method as recited in claim 59, wherein said autologous hematopoietic stem cells are obtained from peripheral blood.

62. The method as recited in claim 59, wherein said autologous hematopoietic stem cells are obtained from bone marrow.

10

63. The method as recited in claim 56, wherein said erythroid progenitor cells are non-autologous stem cells.

15 64. The method as recited in claim 56, wherein at least one of said agents is present in human cord serum.

65. The method as recited in claim 56, wherein at least one of said agents is transforming growth factor beta (TGF-beta).

20 66. The method as recited in claim 56, wherein said step of introducing said treated cells into said subject occurs immediately upon the removal of said treated cells from said from said culture.

25 67. The method as recited in claim 56, wherein said culture is provided as a preserved culture.

68. The method as recited in claim 67, wherein said culture is provided as a cryopreserved culture.

69. The method as recited in claim 56, wherein said culture is provided *ex vivo*.

5

70. The method as recited in claim 56, wherein said agent is a cytokine.

71. The method as recited in claim 56, wherein said subject is a mammal.

10

72. The method as recited in claim 56, wherein said subject is a human.

73. A method for the stimulation of HbF production *in vivo*, the method comprising the step of injecting into a subject having a β -globin disorder, an agent capable of modifying fetal HbF cell proportions.

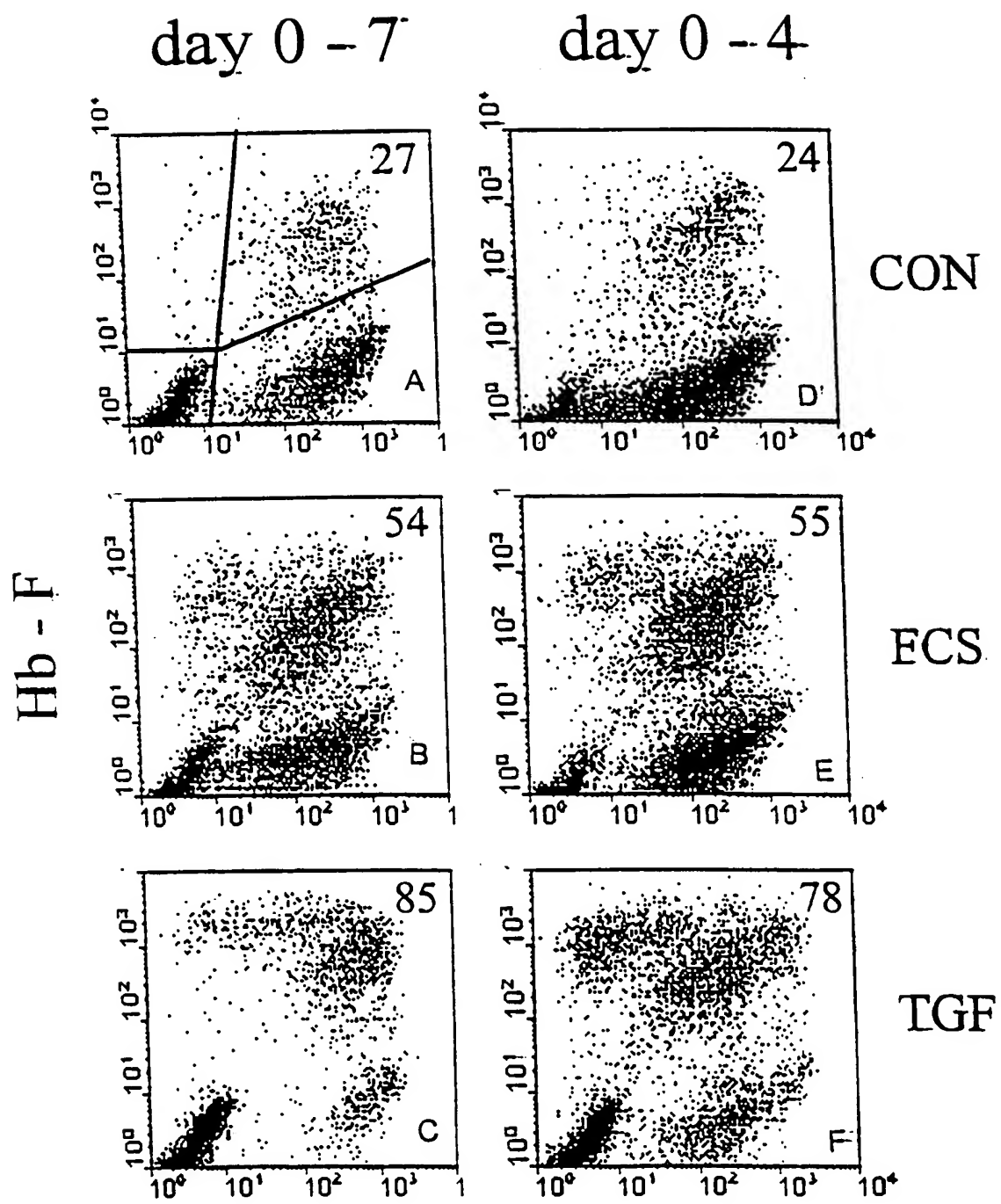
15

74. The method as recited in claim 73, wherein said agent comprises transforming growth factor beta (TGF-beta).

75. A method for the activation of latent TGF-beta in a subject, the method which
20 comprises administering to said subject one or more agents in an amount sufficient to activate latent TGF-beta in said subject.

76. A method for monitoring a response to treatment in patients with β -hemoglobinopathies which comprises generating flow cytometric profiles of correlated
25 contents of different types of hemoglobin present in nucleated or non-nucleated red blood cells of a subject.

77. The method as recited in claim 76, wherein said flow cytometric profiles facilitate in vitro or in vivo observation of erythropoiesis of said subject.



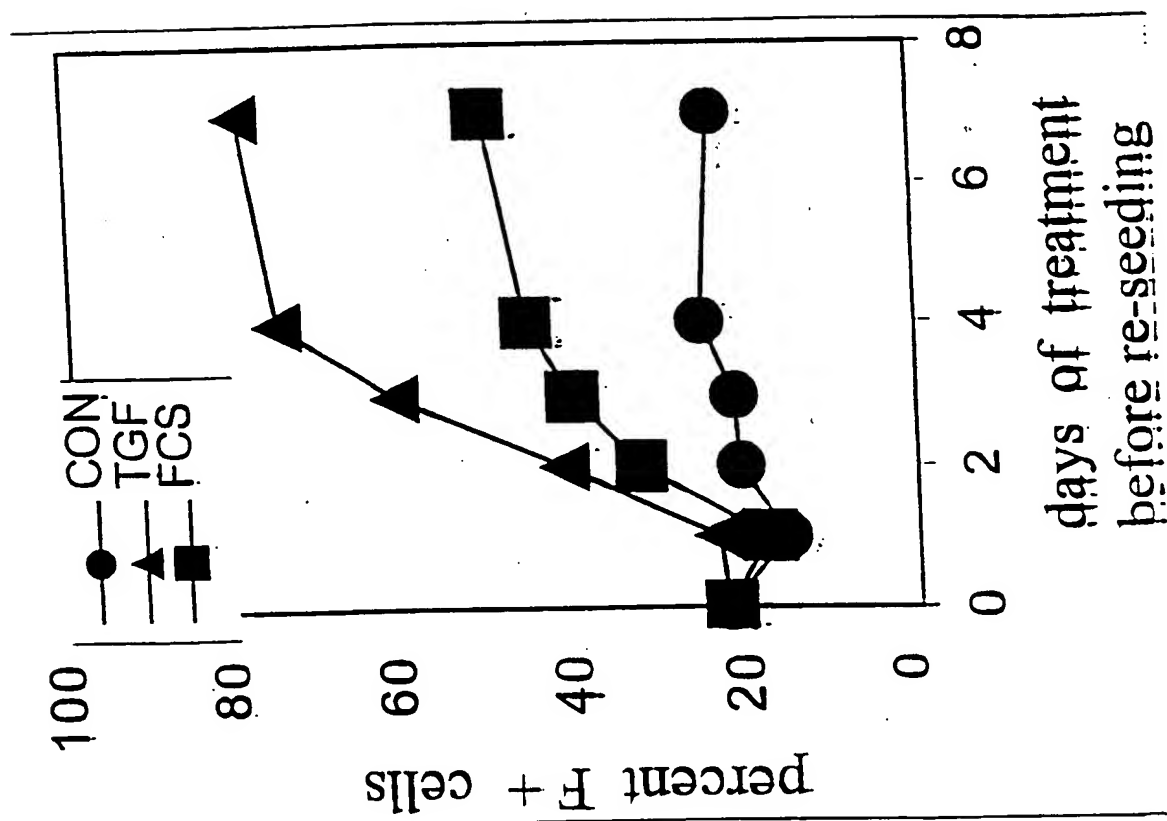
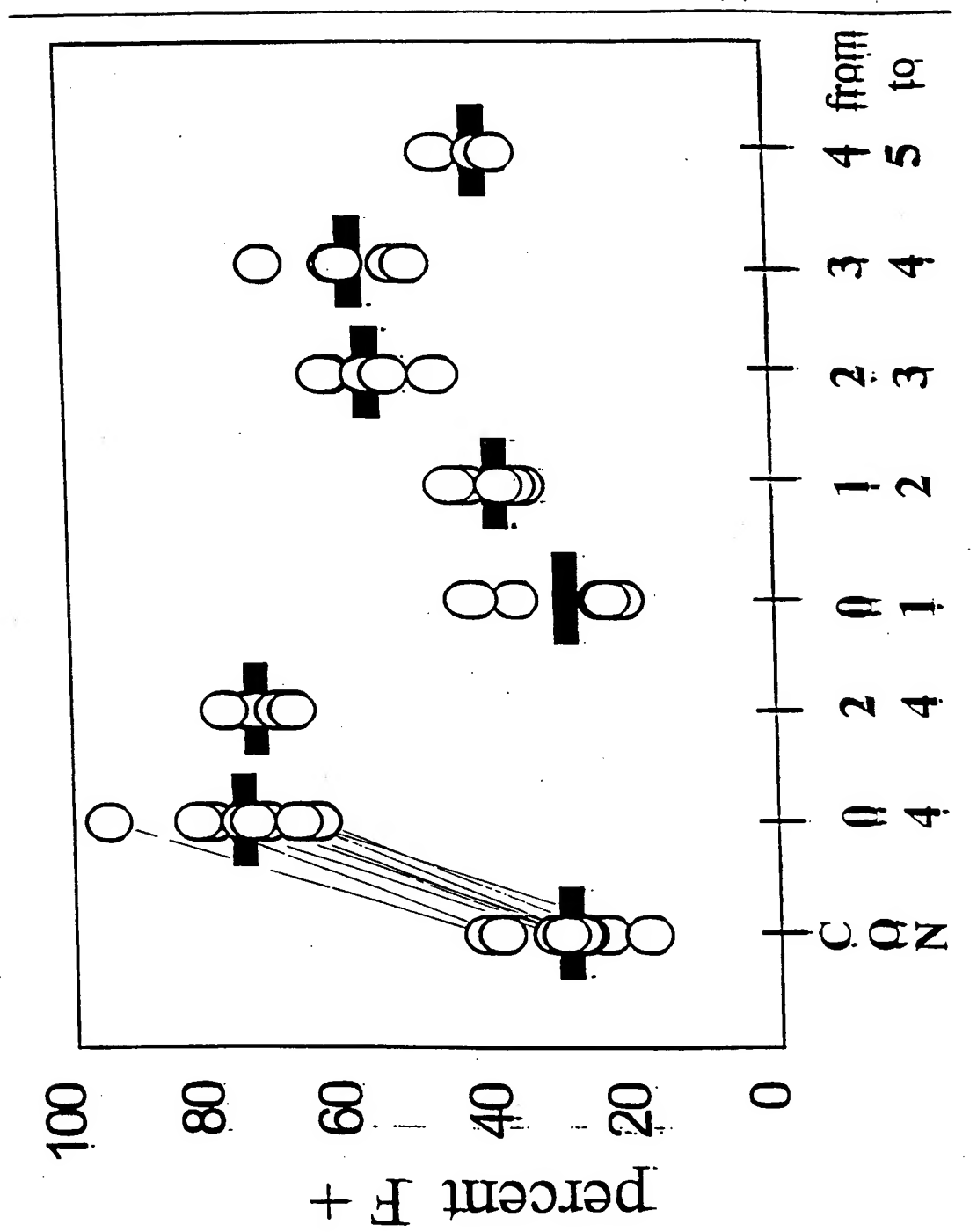


Fig. 2

Fig. 3



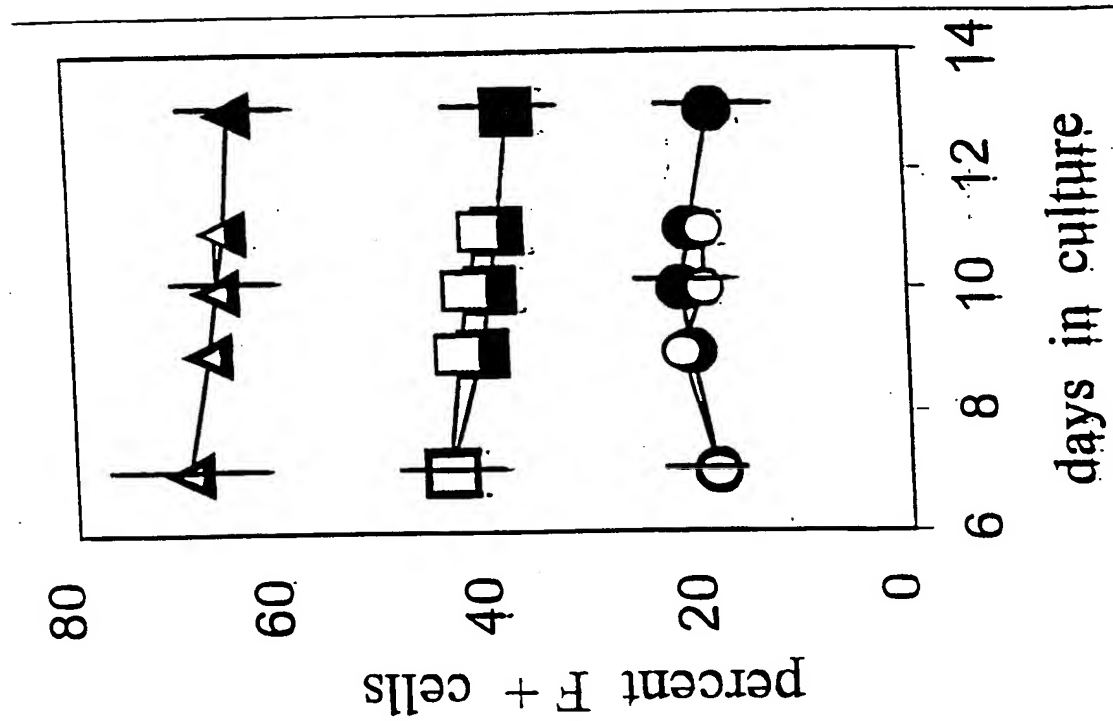
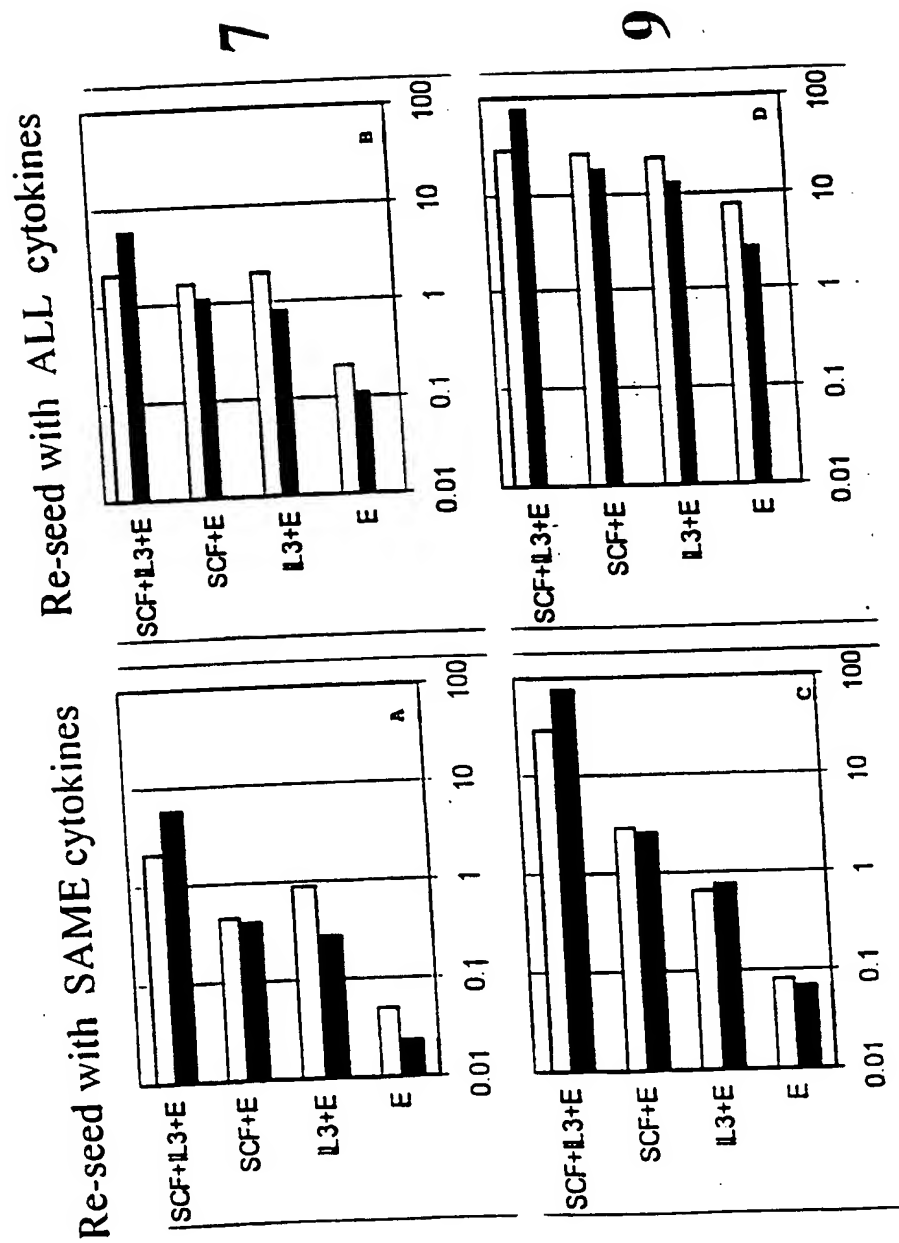


Fig. 4



சின்

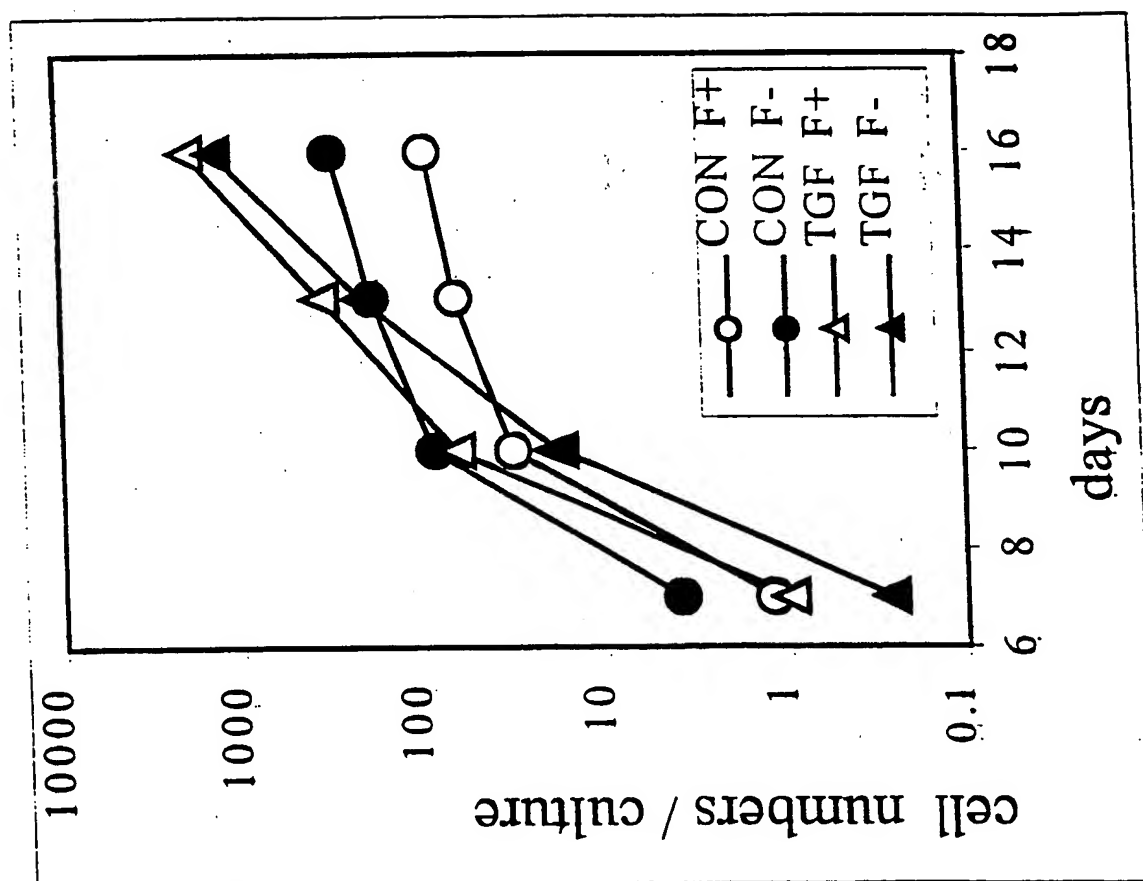


Fig. 5A

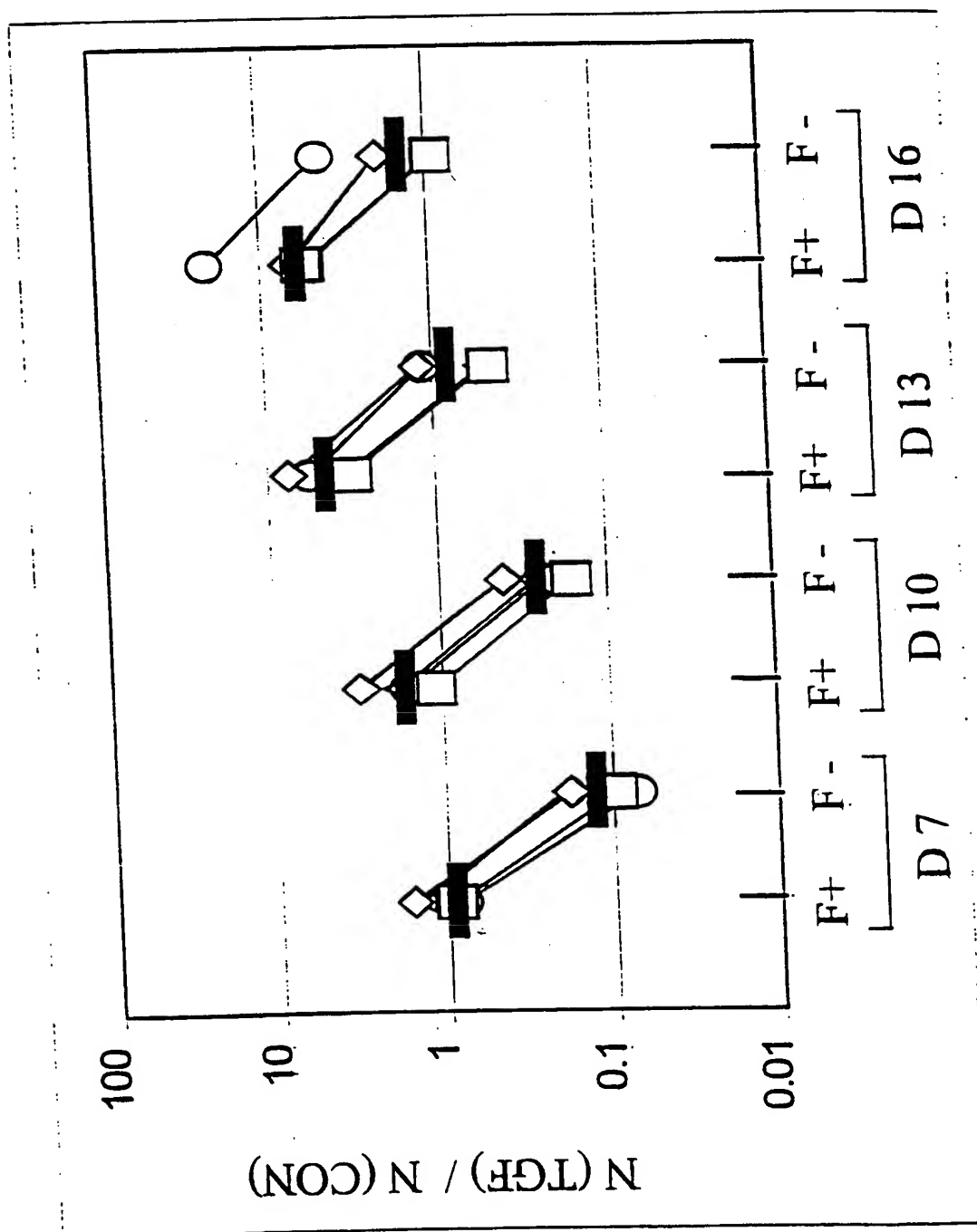


Fig. 5B

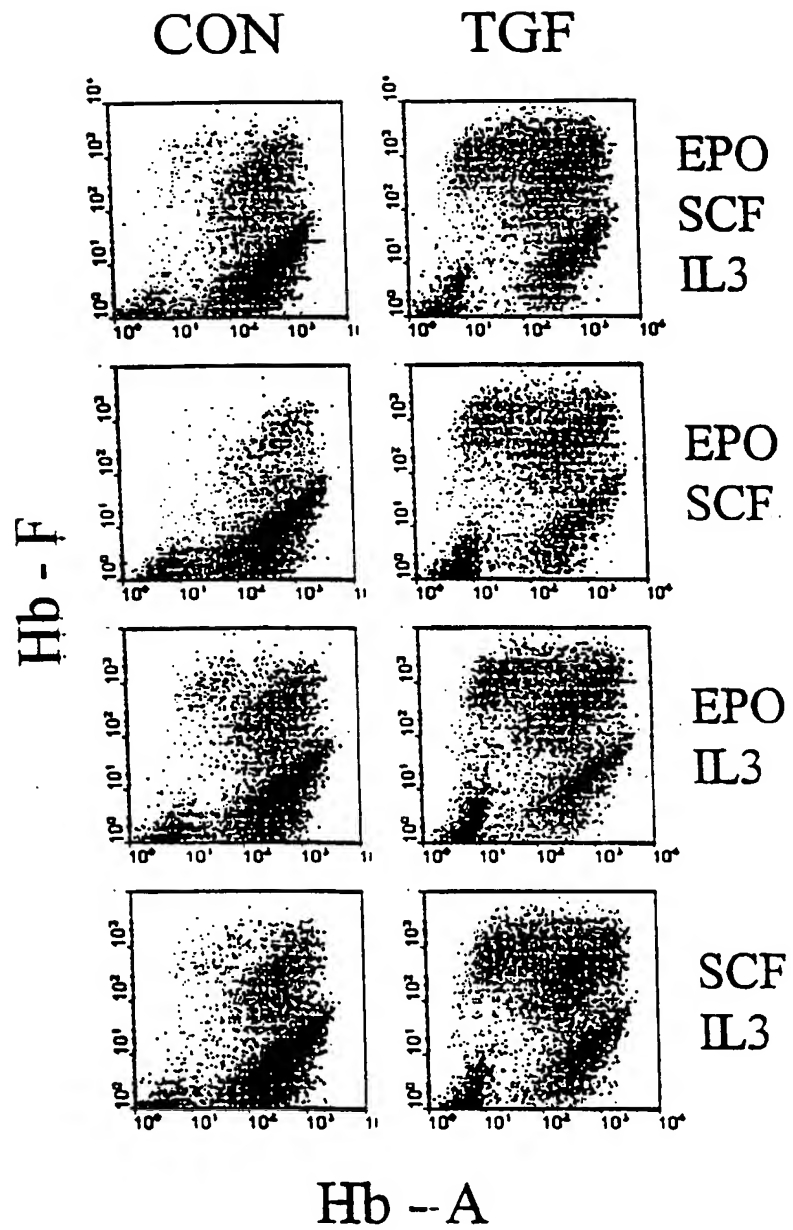
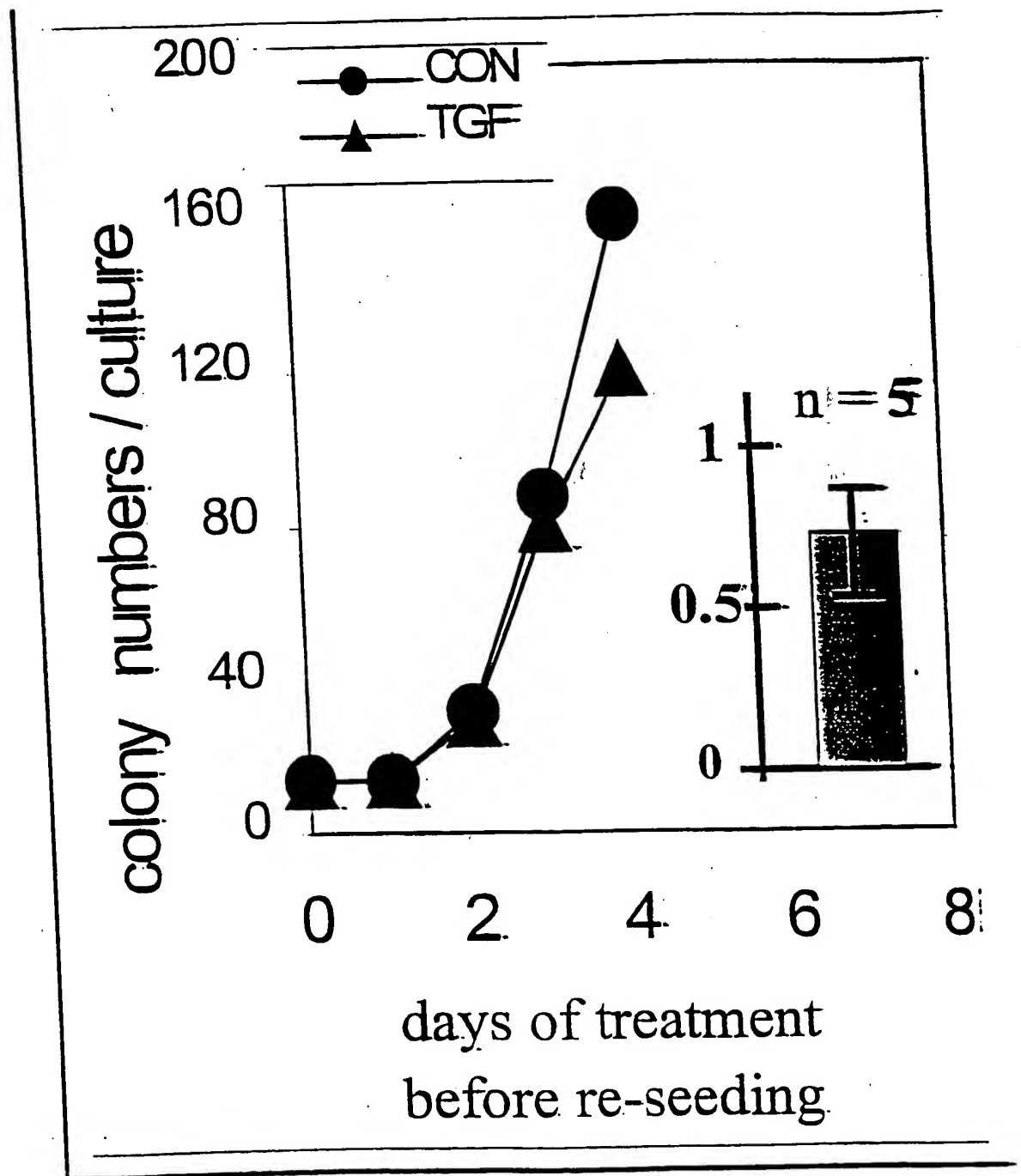
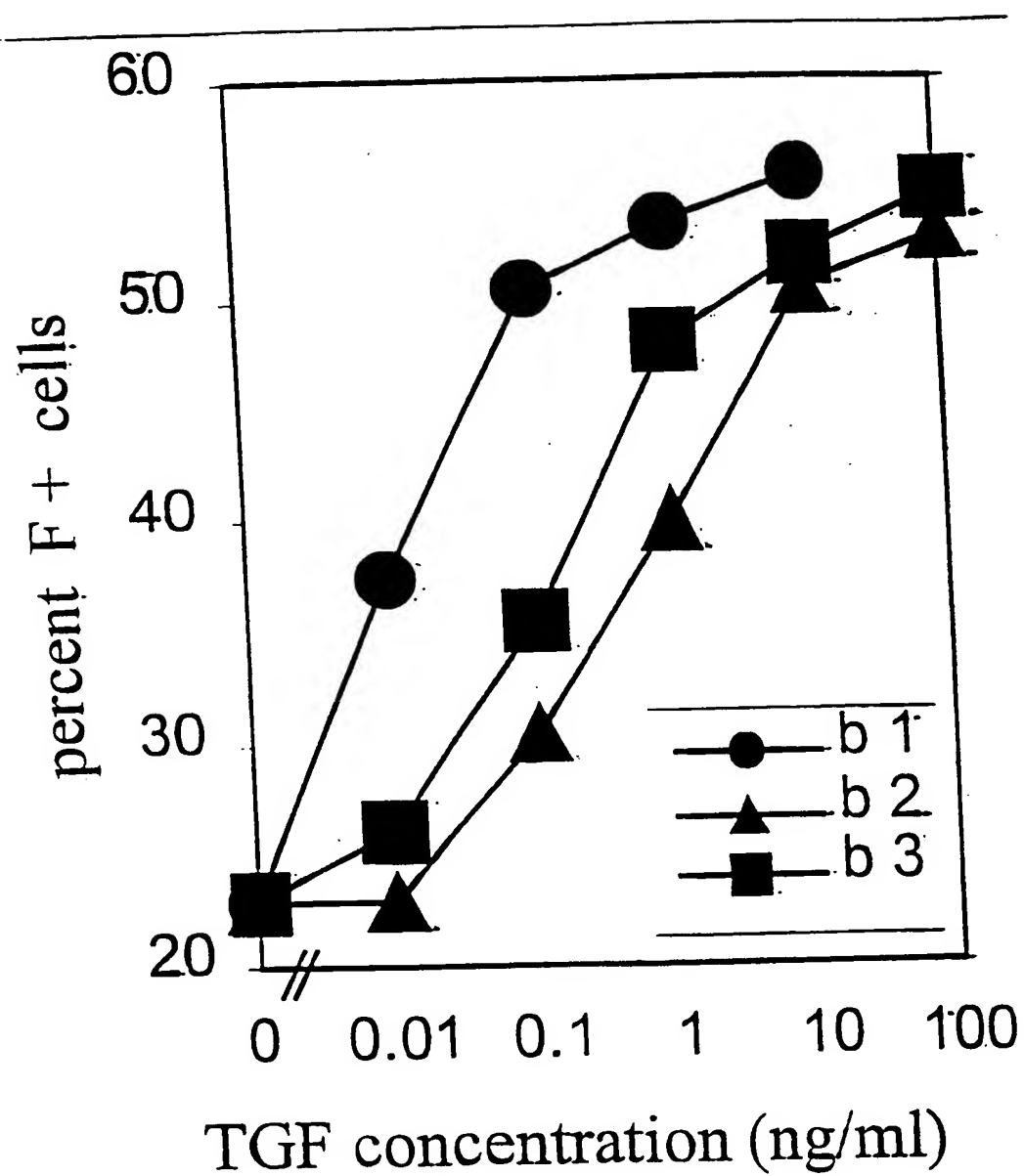


Fig.6





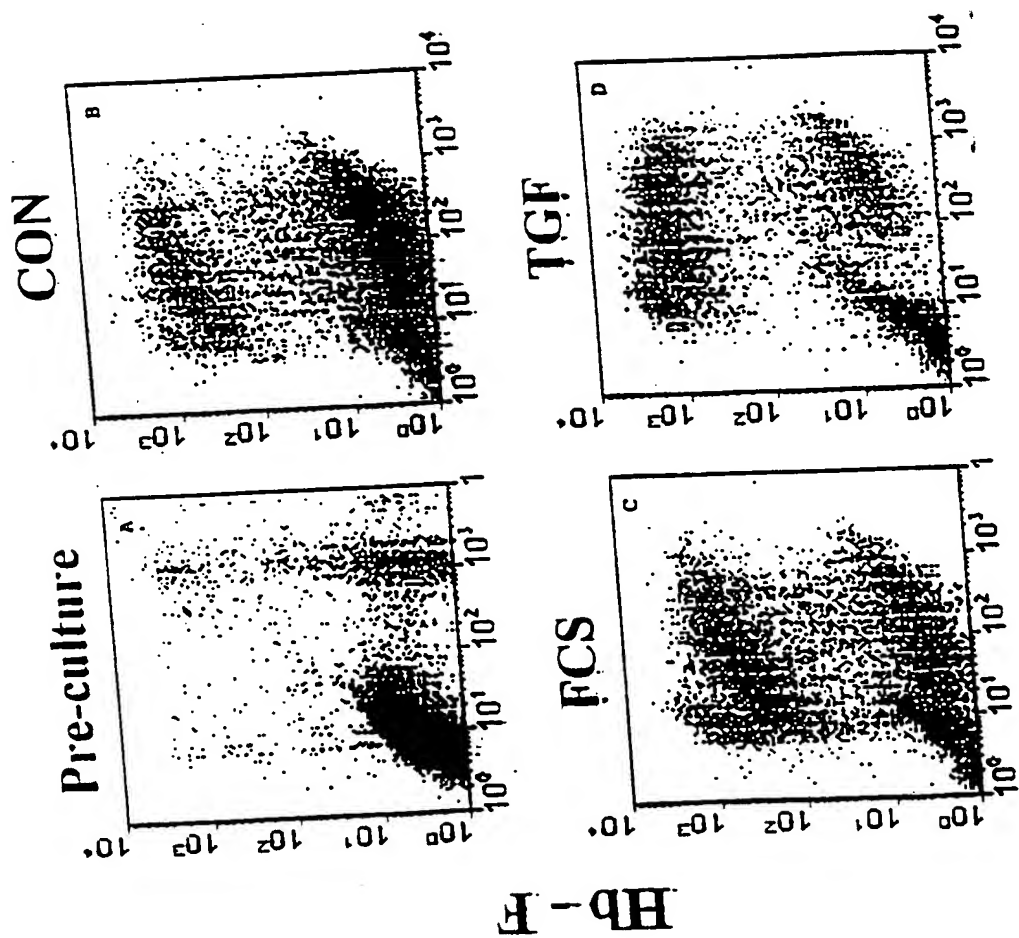


Fig. 9

Hb - E

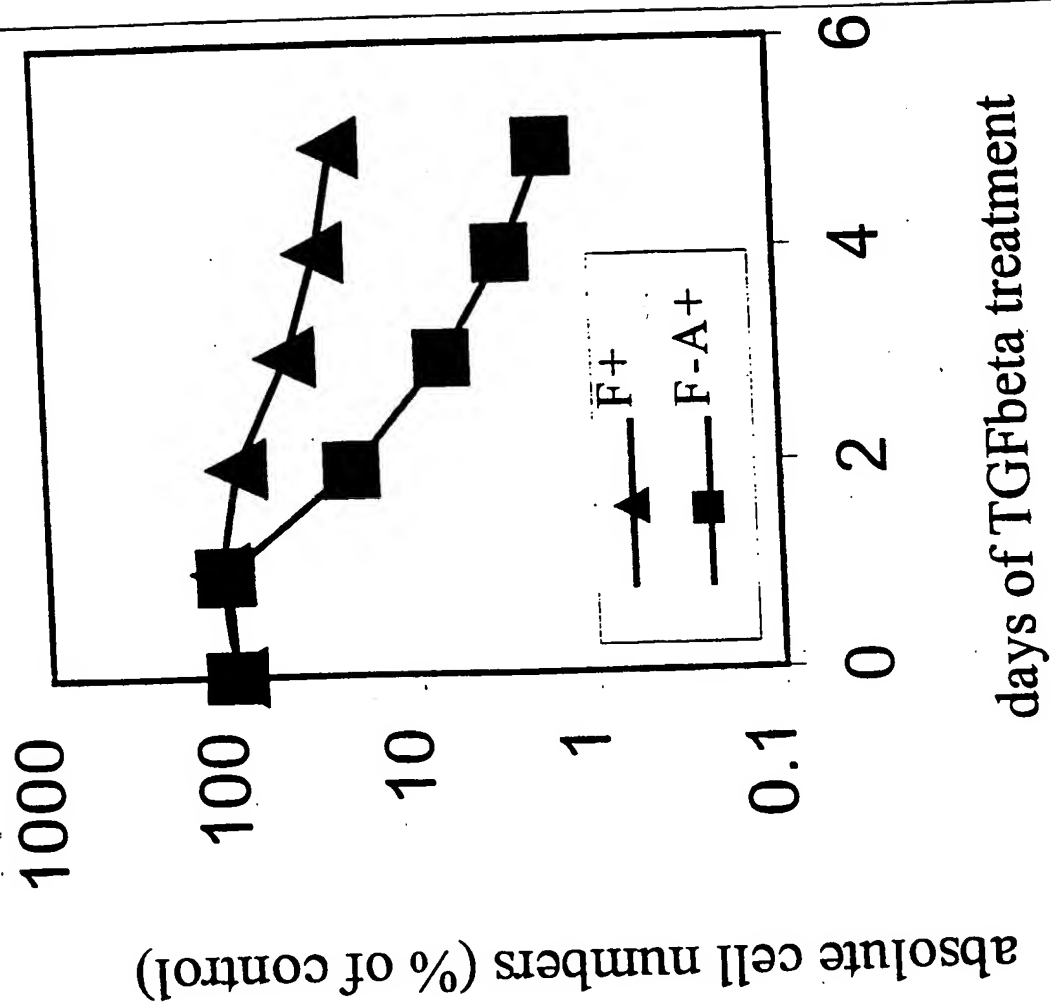


Fig. 10

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N5/06 C07K14/495 A61K38/18 A61K38/19 A61K35/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. GABBIANELLI ET AL., : "Reactivation of HbF synthesis in normal adult erythroid bursts by IL-3" BRITISH JOURNAL OF HAEMATOLOGY, vol. 74, no. 1, January 1990 (1990-01), pages 114-117, XP000944719 the whole document, in particular page 116 right column to page 117 left column	1,2, 15-20, 32-37, 50-54, 56,69-73
X	T. UMEMURA ET AL., : "Effects of interleukin-3 and erythropoietin on in vivo erythropoiesis and F-cell formation in primates" BLOOD, vol. 74, no. 5, October 1989 (1989-10), pages 1571-1576, XP000944732 the whole document --- -/--	1-3,16, 17, 19-21, 33,37, 38,51, 52,54, 70,71,73

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- * & * document member of the same patent family

Date of the actual completion of the international search

15 December 2000

Date of mailing of the international search report

17. 01. 01

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Julia, P

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>E. FIBACH: "Techniques for studying stimulation of fetal hemoglobin production in human erythroid cultures" HEMOGLOBIN, vol. 22, no. 5-6, 1998, pages 445-458, XP000944639</p> <p>the whole document, in particular page 450, page 455 last paragraph and page 457</p>	<p>1-3, 15-21, 32-38, 50-54, 56, 57, 69-73, 76, 77</p>
X	<p>L. SHAO ET AL., : "Effect of activin A on globin gene expression in purified human erythroid progenitors" BLOOD, vol. 79, no. 3, 1 February 1992 (1992-02-01), pages 773-781, XP000574554 the whole document</p>	<p>1, 2, 11, 15-20, 29, 32-37, 46, 50-56, 65, 69-74</p>
X	<p>WO 95 26507 A (UNIV TEXAS) 5 October 1995 (1995-10-05)</p> <p>the whole document, in particular page 16 line 4 to page 17 line 16 and claims</p>	<p>19-28, 32, 33, 35, 36</p>
A	<p>H. CROIZAT AND RL NAGEL: "Circulating cytokines response and the level of erythropoiesis in sickle cell anemia" AMERICAN JOURNAL OF HEMATOLOGY, vol. 60, no. 2, February 1999 (1999-02), pages 105-115, XP000944821 the whole document</p>	<p>1, 2, 11, 19, 20, 29, 37, 46, 54-56, 65, 73, 74</p>
P, X	<p>RM BOHMER ET AL.,: "Selectively increased growth of fetal hemoglobin-expressing adult erythroid progenitors after brief treatment of early progenitors with transforming growth factor beta" BLOOD, vol. 95, no. 9, 1 May 2000 (2000-05-01), pages 2967-2974, XP000944737 the whole document</p>	<p>1-74, 76, 77</p>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/22737

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-77 are directed to methods of treatment and/or diagnostic of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-74

Methods for the treatment of a beta-hemoglobinopathy in a subject, methods for stimulating the production of HbF cells, methods for increasing the proportion of cells expressing and accumulating HbF, methods for stimulating the production of HbF cells in a subject, methods for the treatment of a beta-globin disorder in a subject and methods for the stimulation of HbF production in vivo, wherein all these methods are based on (i) the provision of a culture comprising erythroid progenitor cells and cytokines (TGF-beta) in an amount sufficient to increase the number of fetal hemoglobin producing erythroid cells in said culture, or (ii) the administration of cytokines (TGF-beta) capable of modifying fetal Hb cell proportions.

2. Claim : 75

a method for the activation of latent TGF-beta in a subject, the method which comprises administering to said subject one or more agents in an amount sufficient to activate latent TGF-beta in said subject.

3. Claims: 76-77

a method for monitoring a response treatment in patients with beta-hemoglobinopathies which comprises generating flow cytometric profiles correlated contents of different types of hemoglobin present in nucleated or non-nucleated red blood cells of a subject and said method wherein these flow cytometric profiles facilitate in vitro or in vivo observation of erythropoiesis of said subject.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 2, 10-11,20,28-29,37-50,52-69,71-74 (partial) and 75 (complete)

Present claims 2, 10-11,20, 28-29,37-50,52-69,71-74 (partial) and 75 (complete) relate to methods using an agent or agents which are only defined by reference to a desirable characteristic or property, namely its ability to increase the production of HbF in erythroid progenitor cells (claims 2, 10-11, 20, 28-29,37-50,52-69 and 71-74) and its ability to activate latent TGF-beta (claim 75).

The claims cover all methods using an agent or agents having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such agents, namely "cytokines" and more particularly the transforming growth factor beta (TGF-beta). In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the agent or agents by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the agent or agents being for claims 2, 10-11,20,28-29,37-50,52-69 and 71-74, general "cytokines" or the TGF-beta.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9526507 A	05-10-1995	US 5580724 A	03-12-1996
		AU 684935 B	08-01-1998
		AU 2229895 A	17-10-1995
		CA 2186366 A	05-10-1995
		DE 69501326 D	05-02-1998
		DE 69501326 T	16-04-1998
		EP 0752104 A	08-01-1997
		ES 2111401 T	01-03-1998
		JP 2996513 B	11-01-2000
		JP 9509328 T	22-09-1997
